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actions occur with all constituting amino-acid side chains (*Fig. 2*). That the recovery of tryptophan in the second *Edman*-degradation step is lower than the following methionine (*Fig. 2 A*) is in accordance with the observed high carbene philicity of tryptophan [10]. An apparent limitation of the newly described immobilization system is the low recovery of applied peptide due to the limiting number of available photoactive functions. Experiments designed to augment photolabel density are in progress.

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Abstract. The effects of arachidonic and other *cis*-unsaturated fatty acids were investigated in cultured chromaffin cells from bovine adrenal medulla with respect to Ca^{2+} influx and secretion of catecholamines. As previously shown, arachidonic acid generated during the signal transduction is essential for the fusion of the chromaffin granules with the plasma membrane and by this enables secretion [10–12]. However, if one adds arachidonic acid externally before the agonist, this leads to a partial inhibition of the cholinergic induced secretion. Preincubation with *cis*-unsaturated fatty acids totally blocks the cholinergic induced Ca^{2+} influx but not the Ca^{2+} influx evoked by the voltage gated Ca^{2+}channel. This inhibition is not due to activation of protein kinase C by arachidonic acid, since preincubation with PMA does not block the receptor-dependent Ca^{2+} influx. Preincubation with 5-, 12, 15-HETE and 12-HPETE does not inhibit the receptor-dependent Ca^{2+} influx, indicating that eicosanoids are not responsible for the inhibition. This statement is supported by the fact that BW755C does not reverse the arachidonic acid-evoked blockade of the receptor-dependent Ca^{2+} influx. These results demonstrate that *cis*-unsaturated fatty acids and especially arachidonic acid inhibit the receptor-dependent Ca^{2+} influx by blocking the nicotinic cation channel. The generation of arachidonic acid in the physiological process of the stimulus-response coupling may, thus, contribute to the relaxation, of the cell by preventing further Ca^{2+} influx.

Introduction

Chromaffin cells (CC) from adrenal medulla secrete catecholamines upon stimulation by cholinergic agonists, *e.g.* acetylcholine (ACh) or by depolarization of the plasma mebrane with high K^+ [1]. Stimulation of the cells leads to membrane depolarization and causes a transient increase of membrane permeability to Ca^{2+} (Ca^{2+} influx) which is necessary for the initiation of the secretion [2]. The cells contain nicotinic and muscarinic receptors, both of them beeing activated by ACh [3]. Muscarinic receptors exhibit a higher affinity for ACh [4] and activate the phosphatidylinositol cycle in bovine adrenal medulla without inducing catecholamine secretion [5]. The nicotinic receptor is a heteropentameric complex containing the ligand-binding structure and a cation-selective channel [6]. The supposed physiological sequence of events leading to the large transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) is initiated, when ACh binds to the nicotinic receptor and

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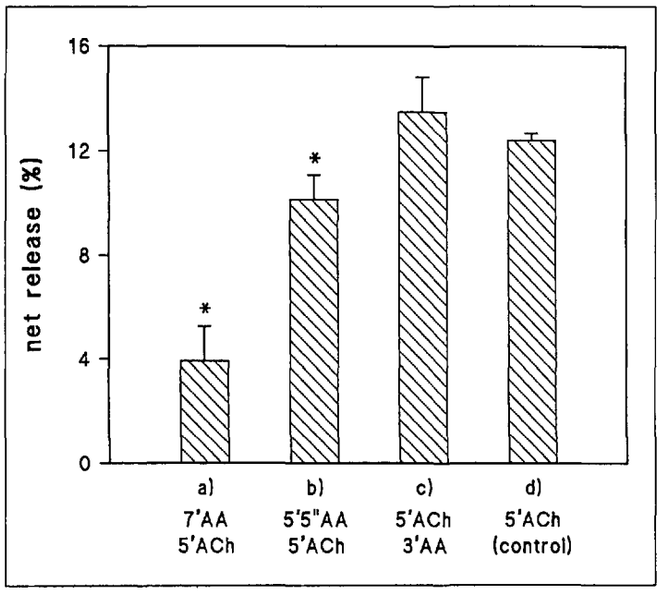

Fig. 1. *Effect of 10* µM *arachidonic acid (AA) on secretion induced by 100* µM *acetylcholine (ACh). a)* Cells were preincubated 2 min in the presence of AA before being stimulated with ACh for 5 min. *b)* Cells were preincubated 5 s in the presence of AA before being stimulated with ACh for 5 min. *c)* Cells were stimulated with ACh for 5 min; at time 2 min AA was added. *d)* Control cells were stimulated with ACh for 5 min. The released catecholamines were calculated in percentage of total catecholamines present in the cells. The results are mean values ± standard deviation of quadruplicates and are representative of seven independent experiments. Analysis of variance gave a significant inhibition of the ACh-stimulated secretion through 2-min preincubation and 5-s preincubation of the cells with AA (*).

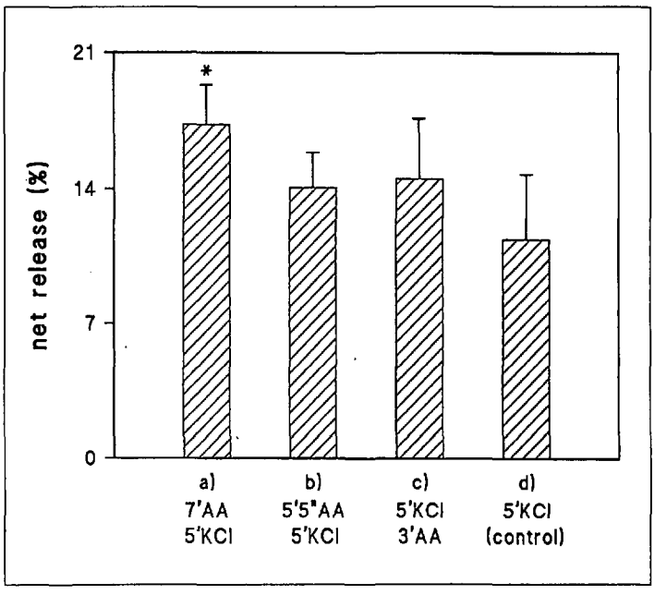

Fig. 2. *Effect of 10* µM *arachidonic acid (AA) on secretion induced by depolarizing the cells with 60 mM KCl. a)* Cells were preincubated 2 min in the presence of AA before being stimulated with KCl for 5 min. *b)* Cells were preincubated 5 s in the presence of AA before being stimulated with KCl for 5 min. *c)* Cells were stimulated with KCl for 5 min; at time 2 min AA was added. *d)* Control cells were stimulated with KCl for 5 min. The released catecholamines were calculated in percentage of total catecholamines present in the cells. The results are mean values ± standard deviation of quadruplicates and are representative of three independent experiments. Analysis of variance gave a significant stimulation of KCl-induced secretion by 2-min preincubation with AA (*).

opens the associated channel, allowing Na^+ entry and local depolarizations. Depolarization then activates adjacent voltage-sensitive Na^+ channels (as an amplifying signal) and voltage-sensitive Ca^{2+} channels producing Ca^{2+} entry [7]. Following stimulation with depolarizing agents, cholinergic receptors undergo a large desensitization which allows recovery of the cells [3] [8]. The original basal level of $[Ca^{2+}]_i$ in CC is reestablished by Na^+/Ca^{2+} exchange and Ca^{2+}-ATPases [9].

We have shown that plasma membranes of bovine CC contain a phosphatidylinositol-specific phospholipase C and a diacylglycerol and monoacylglycerol lipase working in series to liberate arachidonic acid (AA), a process which occurs along with the activated phosphatidylinositol cycle and the Ca^{2+} transient [10]. The role of this AA generation in the signal-release coupling is not yet clear. However, a number of observations indicate the relevance of AA for the release process [11]. We proved that stimulation of bovine CC with exogenous AA (<5 µM) induces secretion of catecholamines, and that one of its lipoxygenase products is essential for the release mechanism [12].

AA and/or its metabolites enhance calcium availability, stimulate phospholipase C, and promote secretion in bovine CC [13] and other tissues [14]. Metabolites of AA generally enhance secretion, while AA itself seems to play an important role in the regulation of cell functions [13]. The following findings support the regulatory role of AA: *1)* Protein kinase C (PKC) is activated by AA and other *cis*-unsaturated fatty acids (UFAs) directly [15–17]. Activation of PKC inhibits voltage-dependent transient opening K^+ and Na^+ channels [18], promotes an active extrusion of Ca^{2+} in pancreatic β-cells [19], inhibits inositol phospholipid metabolism in bovine CC [20], implicating desensitization of muscarinic receptors [21], and desensitizes nicotinic receptors [22]. *2)* AA and other fatty acids directly activate outwardly rectifying K^+ channels in atrial myocytes [23] and smooth muscle cells [24], which leads to hyperpolarization and thereby accelerates desensitization [8]. *3)* AA mobilizes intracellular Ca^{2+} in a number of cell types including bovine CC [13] [25] [26]. *4)* AA and its metabolites inhibit purified Ca^{2+}/calmodulin-dependent protein kinase II [27]. *5)* UFAs inhibit receptor-mediated Ca^{2+} influx in T cells without interfering antibody-receptor binding [28].

In this paper, we show that AA and other UFAs block the receptor-mediated Ca^{2+} influx and secretion in bovine CC, probably by directly blocking the nicotinic channel. This effect is not mediated by metabolites of the lipoxygenase pathway, as shown with the lipoxygenase-inhibitor 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline (BW755C). The physiological relevance of this inhibition for the stimulus-response coupling is discussed.

Experimental

Materials

The lipoxygenase inhibitor BW755C was a generous gift of *The Wellcome Research Laboratories*, Beckenham, England. Collagenase type I, fetal calf serum, neutral red, acetylcholine, nicotine, arachidonic acid, icosapentaenoic acid, γ-linolenic acid, oleic acid, palmitic acid, 5-, 12-, and 15-hydroxyicosatetraenoic acid (5-,12-, and 15-HETE) were obtained from *Sigma*, St. Louis, MO, USA; trypan blue from *Merck AG*, Darmstadt, FRG; *Ham's F12* medium from *Boehringer*, Mannheim, FRG.; *Urografin* 76% from *Schering AG*, Zurich, Switzerland; penicillin, streptomycin, and L-glutamine were from *GIBCO*, Paisley, Scotland; Indo-1/AM from *Molecular Probes*, Eugene, OR, USA; and 12-hydroperoxyicosatetraenoic acid (12-HPETE) from *Calbiochem*, San Diego, CA, USA. The fatty acids, HETEs and 12-HPETE were dissolved in EtOH; Indo-1/AM in DMSO. The final concentration of EtOH or DMSO in the incubation medium was never > 0.3% (v/v) and had no effect on any of the parameters tested.

Test Buffer

If not otherwise indicated, the test buffer for the experiments contained 10 mM glucose, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 133 mM NaCl, 10 mM Tris, and 2.2 mM $CaCl_2$; the pH was adjusted to 7.3 with HCl.

Cell Preparation

Bovine adrenal glands were removed at the local slaughterhouse, and chromaffin cells were prepared according to *Greenberg* and *Zinder* [29] by collagenase digestion of the adrenal medulla in a Ca^{2+}-free *Krebs-Ringer* buffer and purification in a *Urografin* gradient

according to *Wilson* [30]. The cells were transferred into *Ham's F12* culture medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and stored for 2 d at 37° in 95% air, 5% CO_2. Viability of the cells was measured by trypan-blue exclusion. The cells showed a viability of 95–100% directly after isolation of the cells and 60–70% after 2 d of culture. The purity and the number of cells were determined by neutral red staining [31]. The purity was 52–82%; the number of cells isolated of one adrenal gland varied between 2 and 19 millions (according to the season and to the age of the animal). The cells were normally used 48 h after isolation.

Catecholamine Release

The cells were washed with *Krebs-Ringer* buffer containing 2.2 mM $CaCl_2$, centrifuged for 5 min at 500 g, and resuspended in the same buffer at a concentration of 4×10^6 cells/ml. For the release experiments, the cells were incubated at 37° with or without an inhibitor. The reaction was started with the addition of the agonist to the incubated cells. After 5 min, the reaction was stopped by sharp centrifugation at r.t. for 4 s in an *Eppendorf* centrifuge, and the released catecholamines in the supernatant were measured fluorimetrically according to *Lelkes et al.* [32]. The net catecholamine release was obtained by subtracting the catecholamine spontaneously released by control CC from that released by stimulated CC. The released catecholamines were calculated in percentage of total catecholamines present in the CC. *Data analysis*: Each reported value of a release experiment was normally based on 4 independent measurements. Outliers detected with a statistical test according to *Dixon* and *Massey* [33] were eliminated. Data are expressed as means ± standard deviation. For single statistical analysis, a non-paired *Student's t* test was utilized. In studies where multiple comparisons were required, analysis of variance was used. P values < 0.05 were considered significant.

Measurement of Cytosolic Free Calcium Using Indo-1

The cells were washed with test buffer containing 50 µM $CaCl_2$, centrifuged for 5 min, resuspended in the same buffer at a concentration of 4×10^6 cells/ml, and samples were individually incubated for 25 min in the presence of 1–3 µM Indo-1/AM (1–3 nmol of Indo-1/10^6 cells) dispensed in DMSO [34]. Loaded cells were centrifuged for 5 min, resuspended in Ca^{2+}-containing (2.2 mM Ca^{2+}) or Ca^{2+}-free (0 mM Ca^{2+}, 1 mM EDTA) test buffer. The stirred cell suspension in polystyrol cuvettes was equilibrated at 37° and then stimulated by the agonist injected with a microsyringe. An optical multichannel analyzer (OMA) was used for the measurements [35]. Fluorescence was excited by the 350.7- and 356.4-nm emission lines of a tunable krypton laser. Emission spectra were acquired at 350-ms intervals and $[Ca2+]_i$ was calculated on the basis of the ratio of the fluorescence intensities at 405 and 480 nm according to *Grynkiewicz et al.* [34].

Results

Inhibition of Cholinergic Induced Secretion by Preincubation with Arachidonic Acid

The effect of AA on exocytosis was studied by measuring the release of catecholamines in the presence of external AA. We recently reported that, by stimulating the cells with ACh and AA together, the release induced by ACh could be enhanced by AA [12]. In these experiments AA was added to the cells after the addition of ACh. We then could prove that preincubation of CC with 10 µM AA leads to an inhibition of the secretion induced with 100 µM ACh [36]. *Fig. 1* shows the result from a typical experiment where AA was added to CC 2 min or 5 s before ACh or 2 min after ACh. Preincubation of the cells with AA significantly inhibits ACh-induced secretion: by 69% for a 2-min preincubation and by 19% for a 5 s preincubation. The 2 min preincubation of CC with 10 µM AA also leads to an inhibition of the secretion evoked by 100 µM nicotine (data not shown).

It was then interesting to examine the effect of preincubation with AA on depolarization-induced secretion. *Fig. 2* shows that secretion induced by 60 mM KCl is not reduced but is instead significantly enhanced by 2-min preincubation with 10 µM AA. This indicates that the inhibitory effect of AA on secretion in bovine CC is restricted to the cholinergic or more precisely to the nicotinic and not to the voltage-dependent stimulation. As the release of catecholamines is strongly dependent upon $[Ca^{2+}]_i$ elevation, we then focussed on the effect of AA on the nicotinic Ca^{2+} influx.

Blocking of the Nicotinic Channel by Arachidonic Acid and other cis*-Unsaturated Free Fatty Acids*

In agreement with *Negishi et al.* [13], we found that incubating bovine CC with 10 µM free UFAs (AA, icosapentaenoic acid, γ-linolenic acid, oleic acid) alone results in a slight elevation of $[Ca^{2+}]_i$ in the presence of physiologic extracellular Ca^{2+} concentration.

A 2-min preincubation of the cells with the UFAs AA, icosapentaenoic acid, γ-linolenic acid and oleic acid completely blocks the nicotin-induced Ca^{2+} influx, while preincubation with a saturated fatty acid (palmitic acid) does not exert any inhibition (*Fig. 3*). The complete inhibition requires a threshold concentration of >2 µM UFA and already occurs with a 5-s short preincubation. The preincubation with 10 µM AA also completely blocks the ACh-evoked Ca^{2+} influx. When UFAs are added after the cholinergic agonist, the recovery of CC from

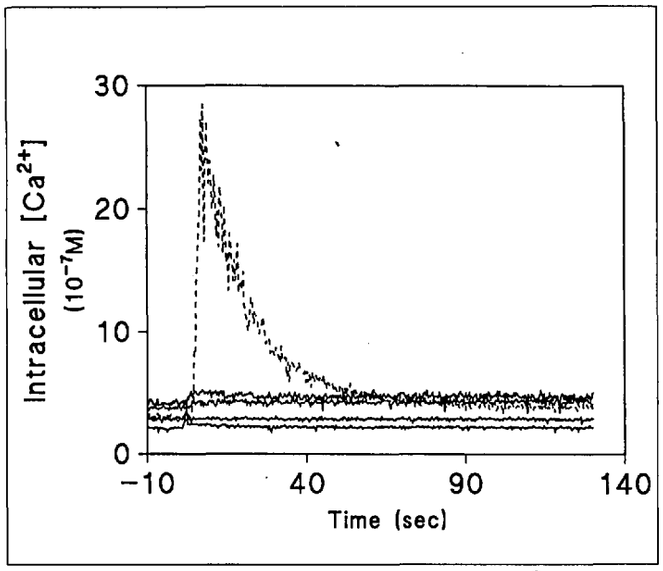

Fig. 3. *Complete inhibition of nicotine-induced Ca^{2+} influx by preincubation with 10* µM *cis-unsaturated fatty acids.* 10^6 cells were loaded with Indo-1/AM, 2 min preincubated with 10 µM AA, icosapentaenoic acid, γ-linolenic acid, oleic acid (solid line) or palmitic acid (dashed line) and stimulated with 100 µM nicotine at time 0. The recordings shown are representative of nine (AA), three (icosapentaenoic acid) and two independent experiments that yielded similar results.

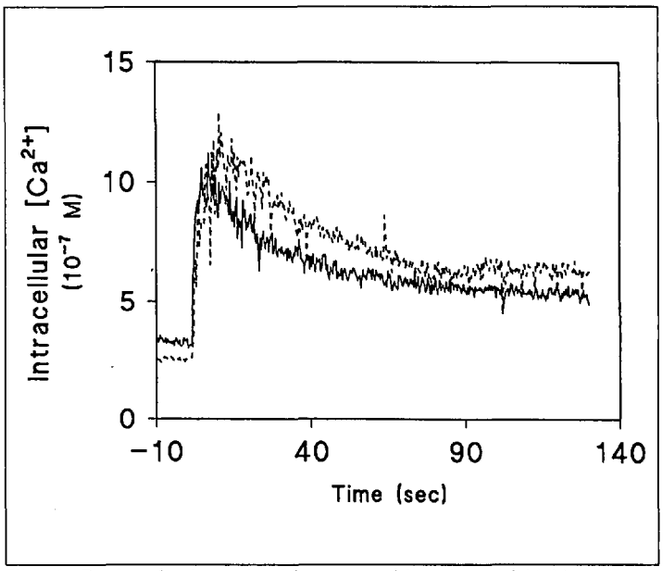

Fig. 4. *Preincubation with 10* µM *arachidonic acid (AA) does not inhibit Ca^{2+} influx induced by depolarizing the cells with 60 mM KCl.* 10^6 cells were loaded with Indo1/AM, 2 min preincubated with 10 µM AA and stimulated with 60 mM KCl (solid line). The control cells were not preincubated with AA (dashed line). KCl was added at time 0. The recordings shown are representative of four independent experiments that yielded similar results.

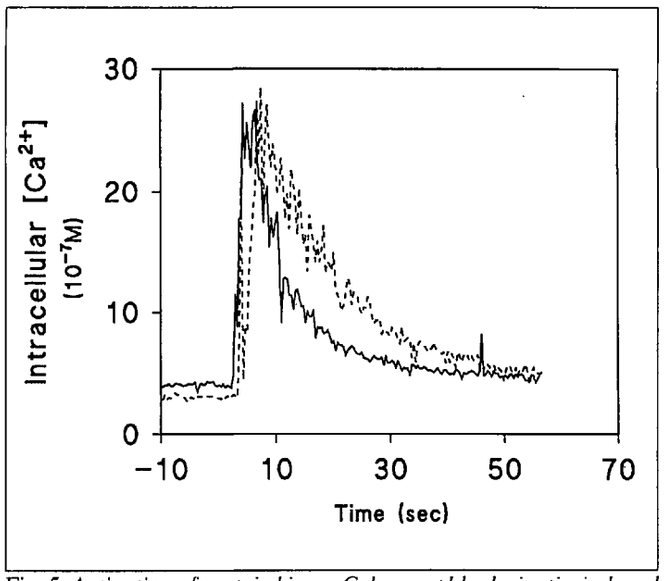

Fig. 5. *Activation of protein kinase C does not block nicotin-induced Ca^{2+} influx.* 10^6 cells were loaded with Indo-1/AM, 2.5 min preincubated with 100 nM protein kinase C activator PMA and stimulated with 100 µM nicotine (solid line). The control cells were not preincubated with PMA (dashed line). Nicotine was added at time 3 s.

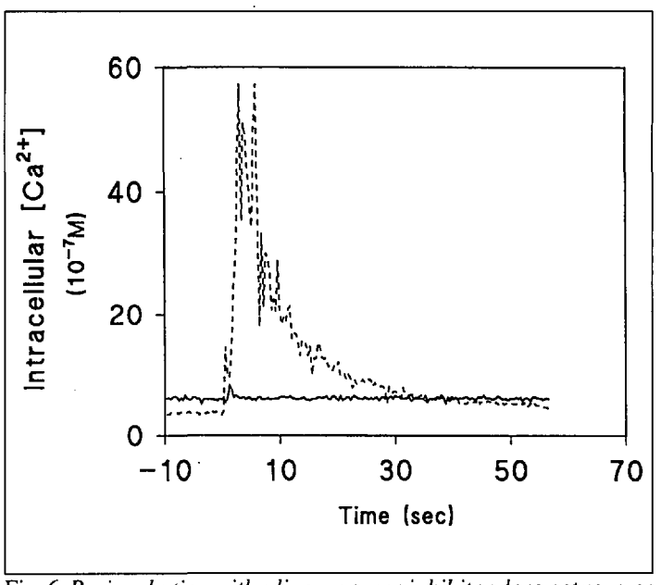

Fig. 6. *Preincubation with a lipoxygenase inhibitor does not reverse the AA-evoked blockade of the nicotine-induced Ca^{2+} influx.* 10^6 cells were loaded with Indo-1/AM, 4 min preincubated with 10 µM lipoxygenase inhbitor BW755C, 2 min preincubated with 10 µM AA and stimulated with 100 µM nicotine (solid line). The control cells were neither preincubated with BW755C nor with AA (dashed line). Nicotine was added at time 0. The recordings shown are representative of two independent experiments that yielded similar results.

elevated $[Ca^{2+}]_i$ occurs faster than in untreated cells (results not shown).

Fig. 4 shows that preincubation (2 min) of CC with 10 µM AA does not inhibit the voltage-dependent Ca^{2+} influx induced by depolarizing the cells with 60 mM KCl as might be supposed from the corresponding secretion experiment.

As UFAs were shown to activate PKC in cell-free systems [16] and bovine CC [17], we tested to see, if such an activation of the PKC was the reason for the blocking of the receptor-mediated Ca^{2+} influx, *e.g.* by phosphorylation of the nicotinic receptors. To investigate an involvement of PKC, the cells were preincubated with PMA, a known activator of PKC. Preincubation with 100 nM PMA for 2.5 min (*Fig. 5*) and 1 µM PMA (data not shown) lowered the Ca^{2+} influx induced by 100 µM nicotine, but by much less compared to UFA-induced inhibition.

Our group recently proved that lipoxygenase products are essential for inducing exocytosis in bovine CC [12]. To examine if the AA-induced inhibition of the nicotinic $[Ca^{2+}]_i$ channel was due to its lipoxygenase products, the cells were pretreated with the lipoxygenase inhibitor BW755C. *Fig. 6* shows that pretreatement (4 min) of CC with 10 µM BW755C – a concentration sufficient to inhibit the ACh-induced secretion by 50% [12] – does not affect the AA-induced $[Ca^{2+}]_i$ elevation or the AA-induced blockade of the nicotinic Ca^{2+} influx, indicating that lipoxygenase products are not involved in this inhibition.

Further evidence that lipoxygenase products do not exert an inhibition on the receptor-mediated Ca^{2+} influx was obtained by preincubating the cells with 100 nM 5-, 12-, and 15-HETE for 2.5 min. This preincubation did not inhibit the nicotine-induced Ca^{2+} influx. In addition, the receptor-mediated Ca^{2+} influx was also not inhibited by preincubation of the cells with 1 µM 5-HETE and 500 nM 12-HPETE (data not shown).

Discussion

Elevation of $[Ca^{2+}]_i$ was evoked by adding 10 µM UFAs to the cells. This is consistent with the finding of *Negishi et al.* [13], who showed that addition of 10 µM AA to bovine CC induced a rapid rise of $[Ca^{2+}]_i$ (from 120 to 400 nM) which was mainly ascribable to the entry of extracellular Ca^{2+}. The small increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (data not shown) may be due to intracellular Ca^{2+} mobilization by UFAs, as previously reported to occur in the endoplasmatic reticulum of T cells [25].

Preincubation (5–150 s) of CC with 10 µM AA and other free *cis*-UFAs results in a total blockade of the receptor-mediated Ca^{2+} influx (*Fig. 3*). Partial inhibition of the secretion could be shown for AA as well (*Fig. 1*). In contrast, this preincubation inhibits neither the Ca^{2+} influx (*Fig. 4*) nor the secretion evoked by depolarizing the cells with 60 mM KCl (*Fig. 2*). This means that the voltage-gated Ca^{2+} channels are not affected. The inhibition is not due to activation of PKC, since preincubation of the cells with PMA had no inhibitory effect on the receptor-mediated Ca^{2+} influx (*Fig. 5*). A similar result was found by *Chow et al.* [28] in T cells, where adding 3–12 µM UFAs inhibited the agonist-evoked augmented $[Ca^{2+}]_i$ level by blocking the influx of bivalent cations. As bovine serum albumin could reverse this effect by binding the UFAs, they claimed that UFAs exert their inhibitory effect on the outer cell membrane, presumably by binding to surface proteins such as the Ca^{2+} channel itself or some component(s) of the channel. Here, we show that voltage-dependent Ca^{2+} influx is not affected by UFAs, clearly indicating that only Ca^{2+} influx through receptor-operated channels is inhibited by these substances. Since UFAs are hydrophobic and have a high affinity for binding to proteins, it is likely that they bind to the nicotinic receptor and/or the associated channel proteins. UFAs do not interfere with the agonist-receptor binding in T cells [28], so of the two possibilities, binding to the nicotinic channel proteins seems the most likely. A direct block of the cation-sensitive nicotinic channel by UFAs also inhibits influx of Na^+ thus preventing depolarization of the plasma membrane and Ca^{2+} influx through voltage-dependent Ca^{2+} channels. The short preincubation time needed for the inhibition of the receptor-mediated Ca^{2+} influx (5 s) may also account for a direct blockade of the nicotinic channel.

Fatty acids have been shown to activate outwardly rectifying K^+ channels in atrial myocytes and smooth muscle cells [23] [24], leading to hyperpolarization of the cells. Hyperpolarization would render more difficult or even prevent depolarization of the cells during normal stimulus-response coupling. UFAs also induce a hyperpolarization in bovine CC [37]. However, this may not account for the inhibition of the receptor-

mediated Ca^{2+} influx: first, a hyperpolarization-mediated block of Ca^{2+} influx does not affect the Ca^{2+} influx and secretion induced by depolarizing the cells with high K^+. Second, hyperpolarization of bovine CC with the K^+-selective ionophore valinomycin does not prevent nicotine-induced Ca^{2+} influx [37].

The results of the present study confirm another important role for AA in the stimulus-response coupling. Endogenous AA and other UFAs generated in the physiological process through the action of a phosphatidylinositol-specific phospholipase C and the diacylglycerol-monoacylglycerol pathway and/or the action of a phospholipase A_2 exert two different, opposed roles. On the one hand AA mediates the stimulation of the cellular response through activating phospholipase C [13] and PKC [17], mobilizing intracellular Ca^{2+} [13] and inducing fusion [38] and secretion [12]. This enhancing effect occurs downstream of signal-release coupling at a moment where the Ca^{2+} channels already have been opened. On the other hand, AA causes relaxation of bovine CC by directly blocking receptor-dependent Ca^{2+} channels and, thus, desensitizsing the cell for prolonged or repeated stimulation. Lipoxygenase products are essential for the release mechanism [12], but they do not block the nicotinic channel (*Fig. 6*, see results).

The time factor decides about the role for AA in the physiological process of signal transduction. Early in the signal transduction AA displays a stimulating effect. In contrast late in the signal-response coupling – when the receptor-mediated Ca^{2+} influx already occurred – AA exerts an inhibiting role and introduces the relaxation of the cell.

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Molekularbiologische Methoden in der Lebensmittelanalytik

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Abstract. Recombinant DNA methods have been rareley used for the analysis of food samples. Since the introduction of the polymerase chain reaction in 1985, DNA analysis of food samples has become a practicable approach. This technique is capable of producing an exponential enrichment of a specific DNA sequence, greatly facilitating subsequent analytical procedures. Detection of infectious agents, identification of irradiated food, and determination of identity and contaminations are potential applications in food analysis. We develop PCR protocols for the detection of *Listeria monocytogenes* and *Escherichia coli* in food and clinical samples. The detection of wheat contamination in glutenfree products, which are intended for coeliac patients is another application currently under investigation.

Einleitung

Fortschritte in den Methoden der Molekularbiologie und der Biotechnologie führten in den letzten Jahren zu stark erweiterten Möglichkeiten für die sogenannte DNA Diagnostik [1]. Fundamental für dieses Gebiet sind die Definition von Nukleinsäure-Sequenzen, die sich als zu bestimmende Parameter eignen, und die Entwicklung von Methoden, die es erlauben, die spezifischen DNA-Sequenzen zu erfassen. Neben Hybridisierungstechniken steht heute die Polymerase-Kettenreaktion (Polymerase Chain Reaction, PCR) im Zentrum. Die PCR ist

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